(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 September 2002 (26.09.2002)

PCT

(10) International Publication Number
WO 02/074330 A2

(51) International Patent Classification[7]: A61K 39/00

(21) International Application Number: PCT/GB02/01323

(22) International Filing Date: 20 March 2002 (20.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0106985.5 20 March 2001 (20.03.2001) GB

(71) Applicant *(for all designated States except US)*: **STANFORD ROOK LIMITED** [GB/GB]; 22 Melton Street, London NW1 2BW (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROOK, Graham, Arthur, William, Prof.** [GB/GB]; 29 Belitha Villas, London N1 1PE (GB). **BRUNET, Laura, Cristina, Rosa, Dr.** [IT/GB]; 63 Harwater Drive, Loughton, Essex, IG10 1LP (GB).

(74) Agents: **MCCLUSKIE, Gail, Wilson** et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5JJ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/074330 A2

(54) Title: IMMUNOTHERAPEUTIC AGENT

(57) Abstract: The present invention relates to mycobacterial extracts comprising lipids, glycolipids and/or carbohydrates and their use in the treatment of autoimmune disease.

IMMUNOTHERAPEUTIC AGENT

Field of the Invention

The present invention relates to mycobacterial extracts comprising lipids, glycolipids and/or carbohydrates and their use in the treatment of autoimmune disease.

Background of the Invention

Autoimmune diseases occur where the individual's immune system attacks their own tissues. Thus they can be defined as diseases where the tissue damage or tissue malfunction is attributable to recognition of a component or components of that tissue by lymphocytes or antibodies. The presentation and nature of autoimmune diseases is inevitably diverse. Diseases generally considered to have an autoimmune origin include type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus.

Several of the most disabling autoimmune diseases of man are Th1-mediated and are increasing in frequency. Multiple sclerosis and type 1 diabetes are particular examples. Current therapies have tended to focus upon attempts to deviate the Th1 response to the autoimmunogen towards Th2, in the belief that Th2 lymphocytes are non-pathogenic in this context, and may, because of the view that there is an inverse relationahip between Th1 and Th2, lead to decreased inflammation. This strategy is proving dangerous, because Th2 effector cells do not always downregulate Th1 activity and may synergise with it. In the experimental allergic encephalitis (EAE) model (Lafaille *et al.*, J Exp Med 186: 307-312 (1997)) and in the induction of diabetes in non-obese diabetic (NOD) mice (Pakala *et al.*, J Exp Med 186: 299-306 (1997)), both of which are primarily Th1-mediated, a superimposed Th2 response can make the situation worse (Lafaille *et al.* (1997); Pakala *et al.* (1997)).

Moreover if the immune response is incorrectly regulated, switching the incorrectly regulated Th1 response to Th2 may merely result in an incorrectly regulated Th2 response that causes a different but equally dangerous disease (Baxter *et al.*, Immunology 83: 227-231 (1994); Genain *et al.*, Science 274: 2054-2057 (1996)). For example, in a treatment trial in human multiple sclerosis (MS), the

disease changed in nature without improvement from the patients' point of view, and autoimmune thyroid disease also appeared, superimposed upon the modified MS (Coles *et al.*, Lancet 354: 1691-1695 (1999); Coles *et al.*, Ann Neurol 46: 296-304 (1999)). The solution to this dilemma is to distinguish between Th2 effector cells, which are potentially dangerous, and Th2-like regulatory cells.

Regulatory cells that can suppress inflammation mediated by autoimmunity have been characterised. It is not clear whether they constitute several different cell types or whether they are variants of a single regulatory cell lineage. A wide variety of organ-specific autoimmune disorders can be shown to be controlled by CD25+ T cells that also express mRNA for IL-4, TGF-β and IL-10 (Seddon & Mason, Immunol Today 21: 95-99 (2000)). They are likely to be related to the IL-10 secreting Tr1 cells that can downregulate the Th1-mediated inflammation in models of inflammatory bowel disease.(Groux *et al.*, Nature 389: 737-742 (1997)). These cells are found in man and for example, they can inhibit the nickel-specific Th1 responses of nickel-reactive individuals (Cavani *et al.*, J Invest Dermatol 114: 295-302 (2000)). They may also be related to Th3 cells that secrete TGF-β and IL-10 (Fukaura *et al.*, J Clin Invest 98: 70-77 (1996)). These are readily derived from human peripheral blood (Kitani *et al.*, J Immunol 165: 691-702 (2000)). They can inhibit both Th1-mediated (Cavani *et al.* (2000); Fukaura *et al.* (1996)) and Th2-mediated autoimmunity (Bridoux *et al.*, J Exp Med 185: 1769-1775 (1997)).

Mycobacteria are powerful immunological adjuvants. That is to say that when mixed with other antigens they enhance the immune response to these antigens. The immune response that is evoked by mycobacteria is very strongly biased towards a Th1 cytokine profile. This is true whether the organisms are used killed and incorporated into oil (as in Complete Freund's Adjuvant (CFA)) or used alive as in vaccination with Bacillus Calmette Guerin (BCG) or following natural infection with *M. tuberculosis*. This Th1 bias is easily demonstrated using peripheral blood mononuclear cells from normal human donors, which preferentially release Th1 cytokines such as interferon gamma (IFN-γ) in response to mycobacterial antigen, showing that exposure to this genus has primed a Th1 response (Del-Prete *et al.*, J. Clin Invest 88 346-350 (1991)). Mycobacteria do this largely because they trigger release of interleukin 12 (IL-12) which drives the response towards Th1, and they

simultaneously impose this Th1 bias on the response to any antigen that is injected with the mycobacterium or emulsified in the CFA (Del-Prete *et al.*, (1991); Romagnani, Immunology Today 13 379-383 (1992)).

Mycobacterium vaccae has particularly potent Th1-adjuvant properties that are manifested even when the organism is used killed, and without incorporation into an oil phase (Abou-Zeid *et al.*, Infect Immun 65 1856-1862 (1997)). It evokes a powerful Th1 response to its own antigens and to antigens associated with it (Abou-Zeid *et al.*, 1997). Similarly if it is injected subcutaneously into the site of previous ovalbumin injections, a killed preparation of this organism can downregulate a pre-existing allergic state in Balb/c mice (Wang & Rook, Immunology 93 307-313 (1998)).

At least some of the ability of micro-organisms to drive Th1-biased responses is attributable to their ability to induce release of proinflammatory cytokines (such as TNFα) and the pro-Th1 cytokine IL-12 from macrophages, and the Th2-opposing cytokine IFNγ from NK cells (Romagnani, (1992)). *Mycobacterium vaccae* is a very potent inducer of IL-12, as expected of an inducer of Th1 lymphocyte responses.

Summary of the Invention

An unexpected effect of lipids, glycolipids and/or carbohydrates derived from mycobacteria has been found. Such lipids, glycolipids and/or carbohydrates have been found to stimulate the development of an antigen-specific cell population in the spleen of subjects to which they have been administered. These cells have been found to be capable of releasing IL-10 and capable of suppressing dysfunctional immune responses in cell transfer experiments. Further, the inventors have found that, surprisingly, there was no increase in IFNγ, indicating that a Th1 response had not been induced. The mycobacterial extract comprising lipids, glycolipids and/or carbohydrates may therefore be used in the treatment and/or prophylaxis of autoimmune disorders such as type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus.

Accordingly, the present invention provides the use of a mycobacterial extract which extract comprises lipids, glycolipids and/or carbohydrates for the manufacture of a medicament for the treatment of an autoimmune disease. Such

material may be administered to a subject, for example a subject who suffers from an autoimmune disease, in an amount sufficient to relieve the symptoms of the autoimmune disease. In a preferred aspect of the invention, the autoimmune disease to be treated is selected from type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus.

The therapeutic agent of the invention comprises lipids, glycolipids and/or carbohydrates derived from a mycobacterium. In a preferred aspect of the invention the lipids, glycolipids and/or carbohydrates are derived from *Mycobacterium vaccae*. In a further preferred aspect of the invention the extract is substantially free from proteins.

Description of the Figures

Figure 1:

IL-12 release from THP-1 cells induced by culture for 18 hours in the presence of lipid fractions from *Mycobacterium vaccae*.

Figure 2:

A: IL-12 release from normal murine (female BALB/c) spleen cells cultured for 3 days in the presence of lipid fractions from *Mycobacterium vaccae*.

B: IFNγ release from normal murine (female BALB/c) spleen cells cultured for 3 days in the presence of lipid fractions from *Mycobacterium vaccae*.

Figure 3:

A: Protocol used in Example 2.

B: IL-10 release from spleens of mice treated with lipid fractions from *Mycobacterium vaccae* according to the protocol shown in A.

Description of the Invention

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The present invention relates to mycobacterial extracts which comprise lipids, glycolipids and/or carbohydrates, and further relates to the ability of the mycobacterial extracts to promote the development of antigen-specific IL-10 secreting regulatory cells.

Extracts of lipids, glycolipids and/or carbohydrates may be obtained from, for example, a frozen mycobacterial cell paste harvested from solid medium or from mycobacteria grown in liquid medium or in a fermentor. Extracts may be prepared by routine methods known to the person skilled in the art. For example, mycobacteria may be extracted using an organic solvent one or more times to obtain a lipid-containing fraction.

In a preferred aspect of the invention, a mycobacterial extract may be produced by treating the mycobacteria to substantially remove protein content. In a further preferred aspect of the invention, mycobacteria are extracted using a solvent for lipids and/or glycolipids, for example, a non-polar solvent. A suitable non-polar solvent may comprise an organic solvent such as chloroform. In a preferred aspect of the invention the solvent comprises a mixture of chloroform and methanol. In a further preferred aspect of the invention the solvent comprises chloroform and methanol in a ratio of 2:1. Such an extraction will produce a solution fraction with a reduced protein content, and preferably a solution fraction which is substantially free from proteins.

The solid residue from such an extraction may be further refined to extract a carbohydrate component and/or smaller, more polar lipids which were not removed during the earlier extraction. For example, the residue may be resuspended in a solvent, for example in 50% aqueous ethanol. This suspension may be treated to substantially remove protein content. For example, the suspension may be refluxed and the solid, protein-containing material removed. The remaining solution will have a reduced protein content, and preferably, the remaining solution may be substantially free from proteins.

A solution fraction produced from the mycobacteria, and a solution fraction produced from the solid residue may be suitable for use in methods of the present invention. They may be used separately or may be combined.

The combined solutions may be dried by removal of solvent. They may then be further extracted. In a preferred embodiment, they may be extracted in chloroform, methanol and water in a ratio of 10:10:3. The solid residue produced by such a further extraction may be used in the methods of the present invention. The solution fraction of such a further extraction also comprises active material. This solution fraction may be further refined by extraction of undesired material. Undesired material may be extracted by distribution between a polar and non-polar solvent, for example between methanol and petrol. A solution fraction may be further refined by further extractions using organic solvents as described above. Preferably a solution fraction may be further extracted using chloroform and methanol in a ratio of 2:1. A solution fraction may alternatively or additionally be further treated to precipitate an insoluble fraction, for example by treatment of a solution fraction with acetone.

The activity of a mycobacterial extract may readily be established by one skilled in the art using, for example, a method as described in Figure 3A. Briefly, Balb/c mice are pretreated intraperitoneally with 10μg ovalbumin adsorbed onto alum twice at 0 and 12 days. Such a composition of ovalbumin may be produced by any of the methods well known in the art. They are then treated on day 21 with 5μg of the mycobacterial extract or an equivalent volume of saline. Ovalbumin treatment is repeated at days 42 and 54. On days 61 and 63 ovalbumin is administered at a dose of 50 μg intra-tracheally. On day 65, the spleen is removed and cultured. The cultured spleen is then tested for the ability to produce IL-10 in response to allergen *in vitro*. IL-10 may be assayed by any of the methods well known in the art, for example, IL-10 may be assayed using capture ELISA.

An extract may be from a particular species of mycobacterium, for example *Mycobacterium vaccae*, *Mycobacterium fortuitum*, *Mycobacterium dienhoferei*, *Mycobacterium smegmatis*, *Mycobacterium flavescens* or *Mycobacterium phlei*. The mycobacterium is preferably *Mycobacterium vaccae*. An extract may be from a particular strain of mycobacterium. Particularly preferred strains of *Mycobacterium vaccae* include *M. vaccae* R877R (deposited at the National Collection of Type Cultures Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, United Kingdom on February 13th, 1984 under the number NCTC 11659) and ATCC

15483 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA).

Alternatively it may be advantageous and is within the scope of the invention to use lipids, glycolipids and/or carbohydrates derived from more than one species of mycobacterium, or from more than one strain of a mycobacterial species, for example from more than one strain of *Mycobacterium vaccae*.

One aspect of the present invention is the use of the lipids, glycolipids and/or carbohydrates derived from mycobacteria as referred to above in the prevention or treatment of treatment of autoimmune disorders such as type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus. The lipids, glycolipids and/or carbohydrates may be administered to a patient suffering from or subject to autoimmune disease. The treatment may therefore be therapeutic or prophylactic. The condition of a patient suffering from such a disease state can thus be improved. The normal symptoms of a patient subject to an autoimmune disease can thus be prevented.

Lipids, glycolipids and/or carbohydrates obtained as outlined above may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art. For example, a suitable lipid and/or glycolipid may be put into suspension in, for example, a physiological buffer, isotonic saline or water by physical disruption such as ultrasound. Alternatively it may be put into suspension by ultrasound in the presence of a stable carrier protein, for example lipid-free human serum albumin, to which the lipid and/or glycolipid will bind, providing a stable solution. Alternatively the lipid and/or glycolipid may be formulated as slow release pellets following combination with a suitable carrier molecule, for example cholesterol. A suitable carbohydrate that is linked to a lipid or glycolipid may be formulated in the same way as a lipid and/or glycolipid. A suitable carbohydrate not linked to a lipid or glycolipid may be dissolved in, for example, physiological saline or water for injection. The exact nature of a formulation will depend upon several factors including the particular substance to be administered and the desired route of administration. Suitable types of formulation are fully described in Remington's Pharmaceutical Sciences, Mack Publishing

Company, Eastern Pennsylvania, 17th Ed. 1985, the disclosure of which is included herein of its entirety by way of reference.

The pharmaceutical composition comprising lipids, glycolipids and/or carbohydrates may also contain further ingredients such as adjuvants, preservatives, stabilisers etc. It may further comprise other therapeutic agents. It may be supplied in sterile and pyrogen free form, for example as an injectable liquid; in sterile freeze-dried form which is reconstituted prior to use; or as sterile slow-release pellets. The pharmaceutical composition may be supplied as an isotonic liquid. It may be supplied in unit dosage form.

The substances may be administered by enteral or parenteral routes such as via the oral, buccal, anal or topical route, by subcutaneous, intradermal, intravenous, intramuscular or intradermal injection, by aerosol into the airways, or by other appropriate administration routes. Particularly preferred routes of administration are the oral route or by subcutaneous or intramuscular injection. A physician will be able to determine the required route of administration for any particular patient.

A therapeutically effective amount of a lipid, glycolipid and/or carbohydrate is administered to a patient. The dose may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. Multiple doses may be given. A typical individual dose is from about 0.0001 to 200 mg, preferably from about 0.0005 to 0.5 mg, more preferably from about 0.001 to 0.01 mg, according to the activity of the specific lipid, glycolipid and/or carbohydrate preparation, the age, weight and conditions of the subject to be treated, the type and severity of the autoimmune disease and the frequency and route of administration.

The invention will be described with reference to the following Examples, which are intended to be illustrative only and not limiting.

Example 1

Lipids and glycolipids are prominent amongst the microbial components that drive the IL-12 and IFN-γ production, and so bias responses towards Th1 (Almeida

et al., EMBO J 19 1476-1485 (2000); Roach *et al.*, Immunology 85 106-113 (1995)). Lipid fractions isolated from *Mycobacterium vaccae* were therefore tested to find out if they share these properties.

Lipids may be prepared from *Mycobacterium vaccae* as follows:

Starting material; frozen mycobacterial cell paste harvested from solid medium or from material grown in liquid medium or in a fermentor.

Lipid extraction. The paste is dried at 60°C +/- 5°C in a vacuum oven for 75 hrs +/- 20hrs. The paste is then extracted with chloroform:methanol 2:1 (v/v) for 90 mins. For example dried organisms derived from 90 gm of paste may be extracted with 240 ml of chloroform:methanol. The solvent extract, comprising lipids and glycolipids, is saved, and the extraction repeated with another 240 ml of chloroform:methanol. The second batch of solvent extract is pooled with the first. The extracted organisms are then air-dried for 20hrs+/-4 hrs. They are then resuspended in 200 ml of 50% aqueous ethanol and refluxed for 2 hrs. This process produces aqueous ethanol solvent which comprises smaller, more polar lipids which were not extracted by the chloroform:methanol, and is substantially free from proteins. This refluxing stage is performed 3 times, and the solvents pooled.

The chloroform:methanol solvent pools and the aqueous ethanol solvent pools may then be combined before drying *in vacuo*, or the two solvent pools may be dried *in vacuo* separately. The fractions used in the experiments reported below were derived from the combined pools as described below.

Fractionation of lipids/glycolipids;

The material is taken up in chloroform/methanol/water 10:10:3.

Fraction 1 – insoluble components, air-dried, and used as it is.

Fraction 2 – soluble components, distributed between petrol and aqueous methanol.

Fraction 3 – petrol layer produced from fraction 2, air-dried and used without further fractionation.

Fraction 4 – aqueous methanol layer produced from fraction 2, dissolve in chloroform methanol (2:1), acetone added to form precipitate.

Fraction 7 – precipitate produced from fraction 4, air-dried

Fraction 8 – soluble components produced from fraction 4, air-dried

For use *in vitro* the fractions are weighed, dissolved, and aliquotted into glass containers and dried again, so that known quantities are present in the containers. Then tissue culture medium or buffered saline is added containing delipidated human or murine serum albumin. Exposure to ultrasonic disintegration then causes the lipids/glycolipids to stay in suspension in association with the albumin.

Fractions were obtained from *Mycobacterium vaccae* as described above. Lipid/glycolipid fractions 7 and 8 were found to drive IL-12 release from THP-1 monocytoid human cell line (Fig 1), and from normal mouse (Balb/c) spleen cells (Fig 2A). The release of IL-12 was assessed by the following method. THP-1 cells were grown up, treated with DMSO for 24 hours in order to differentiate them. The cells were mixed with the fractions and cultured for 18 hours. The supernatant was then assayed for IL-12 by a capture ELISA method.

Similarly fractions 4 and 7 induced release of IFNγ from normal Balb/c spleen cells (Fig 2B). The release of IFNγ was assessed by the following method. Balb/c spleen cells were taken form the spleens of mice and cultured for 72 hours with the fraction. The supernatant was assayed for IFNγ by a capture ELISA method.

These results suggested that lipid fractions of *Mycobacterium vaccae* would be potent Th1 adjuvants.

Example 2

Since *Mycobacterium vaccae* is a Th1-inducing organism, and since lipid fractions 4, 7 and 8 were potent inducers of the Th1-biasing cytokines IL-12 and IFN□, it was hypothesised that these lipid materials would act as Th1 adjuvants and induce a Th1 response to the antigen in the Balb/c mouse model of ovalbumin-induced, Th2-mediated autoimmune disease. A number of the extracted fractions produced in Example 1 were therefore dried and tested according to the protocol shown in Fig 3A.

Briefly, Balb/c mice were pretreated intraperitoneally with 10μg ovalbumin adsorbed onto alum twice at 0 and 12 days. They were then treated on day 21 with 5μg of the extract or an equivalent volume of saline. Ovalbumin treatment was repeated at days 42 and 54. On days 61 and 63 ovalbumin was administered at a

dose of 50 μg intra-tracheally. On day 65, the spleen was removed and cultured. The cultured spleen was then tested for the ability to produce IL-10. IL-10 was assayed using capture ELISA.

The fractions tested were fraction 4, (which contains fractions 7 and 8) and fraction 1. Fraction 3 which had not shown any biological activity *in vitro* was included as an additional control.

Surprisingly, fractions 4 and 1 failed to drive a Th1 response, and instead caused the animals to develop an antigen-specific cell population in the spleen that released IL-10 when cultured *in vitro* in the presence of ovalbumin. There was no release of IL-10 from cultured spleens of any donors if ovalbumin was not added to the cultures. Therefore the response indicated the presence of antigen-specific IL-10-secreting cells.

Spleen cells from animals that had not been treated with the fractions made no detectable IL-10 in response to ovalbumin (Fig 3B). Moreover there was no increase in IFNγ output in response to ovalbumin, indicating that a Th1 response had not been induced. Similarly the output of IL-5 was not significantly altered, indicating that there was also no increase or decrease in Th2 response. The effects of the lipid fractions were therefore not being mediated by Th1 or Th2. Thus the mode of action of the lipid fractions differs from that of the parent organism which has previously been shown to cause a complete cessation of antigen-induced release of IL-5 (Wang & Rook, 1998). Antigen-specific cells (i.e. T cells) that make interleukin 10 (IL-10) but not IL-5 or IFNγ constitute a population of "regulatory" or "suppressor" T cells (Cavani *et al.*, J Invest Dermatol 114 295-302 (2000); Cottrez *et al.*, 2000; Groux *et al.*, Nature 389 737-742 (1997)). The induction of such cell populations by mycobacterial lipids and glycolipids constitutes a novel finding with multiple uses.

The effect seen with these mycobacterial lipid fractions did not require the lipids to be injected together with the immunogen, or into the same lymphatic drainage area. For example, induction of antigen-specific IL-10-secreting cells was seen in animals immunised by the intraperitoneal route, and treated at an entirely different time point by the subcutaneous route with the lipid fractions alone. This

property enables the use of such fractions to promote regulatory cell development even when the immunogen is unknown, as in an autoimmune disease.

Example 3

The mice used in the following example were female NZBxW F1 mice from different parents, numbering 33 in total. The mice were randomised to minimise risks of stratification.

The experiment was staggered because of mice availability and when the injections could be started. The mice included a first batch of 12 mice, a second batch of 12 mice born 24 days later and a third batch of 9 mice, born 58 days after the first batch.

The mice were given 3 injections at 4-week intervals with the first injection starting at 4 weeks of age. To control for what is known as "cage effect" the mice were grouped such that each set of 3 mice were placed in a cage with one given sterile saline, the second given Fraction 4 (as prepared in Example 1 above) and the third given a preparation of killed whole *M. vaccae*.

The mice were bled at 4 weekly intervals starting from the age of 16 weeks. Weight was measured on a weekly basis starting from 4 weeks of age and proteinuria again measured weekly, starting from the age of 12 weeks.

Normally, this strain of mice would be expected to start to show signs of illness between the ages of 6 to 9 months. This experiment is still in progress, but early data have become available. At about just over 6 months of age, 6 mice in the first batch of 12 mice have high levels of proteinuria (some starting from about the age of 5 months). Three of these mice are from the group given the killed whole *M. vaccae* preparation (75%), 2 from mice given sterile saline (50%) and 1 mouse given Fraction 4 (25%). Three of the mice that developed high levels of proteinuria died about 10 to 14 days later after developing proteinuria. Two of the dead mice were those given the killed whole *M. vaccae* preparation making the fatality rate within this group 50%. The third dead mouse was one that received sterile saline.

The only mouse from the group that received Fraction 4 and had developed proteinuria is still alive and appears well. Proteinuria was observed in this mouse for the first time at aged 146 days. In fact according the assessment we carried out on this mouse at age 188 days the level of proteinuria in this mouse appeared to have

gone down. The mouse is certainly alive about 7 weeks after first showing signs of proteinuria. Two of the dead mice actually shared the cage with this surviving mouse. Therefore, "cage effect" is effectively taken out of the equation in this instance.

In the second batch of 12 mice, 3 mice have proteinuria, 2 of which were given the whole killed *M. vaccae* preparation and one which was given sterile saline. At aged 169 days, there were no fatalities.

IgM and IgG antibody levels to double stranded DNA have been observed. These results have not been extensively analysed, but seem to suggest that mice receiving sterile saline or the whole killed *M. vaccae* preparation have higher levels of autoantibodies.

CLAIMS

1. Use of a mycobacterial extract which extract comprises lipids, glycolipids and/or carbohydrates for the manufacture of a medicament for the treatment of an autoimmune disease.

2. The use according to claim 1, wherein the mycobacterial extract comprises lipids and/or glycolipids.

3. The use according to claim 1, wherein the mycobacterial extract comprises carbohydrates.

4. The use according to any one of the preceding claims, wherein the mycobacterial extract is substantially free from proteins.

5. The use according to any one of the preceding claims, wherein the mycobacterial extract is from *Mycobacterium vaccae.*

6. The use according to any one of the preceding claims, wherein the autoimmune disease is type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis or systemic lupus erythematosus.

7. A method of treating an autoimmune disease which comprises administering to a subject suffering from or subject to autoimmune disease a mycobacterial extract which extract comprises lipids, glycolipids and/or carbohydrates in an amount sufficient to prevent, control or alleviate the symptoms of the autoimmune disease.

8. A method according to claim 7, wherein the mycobacterial extract comprises lipids and/or glycolipids.

9. A method according to claim 7, wherein the mycobacterial extract comprises carbohydrates.

10. A method according to claim 7, wherein the mycobacterial extract is substantially free from proteins.

11. A method according to claim 7, wherein the mycobacterial extract is from *Mycobacterium vaccae*.

12. A method according to claim 7, wherein the autoimmune disease is selected from the group consisting of type 1 diabetes, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus.

13. An extract comprising lipids, glycolipids and/or carbohydrates which extract is derived from *Mycobacterium vaccae* and which is substantially free from proteins.

14. A pharmaceutical composition comprising lipids, glycolipids and/or carbohydrates derived from *Mycobacterium vaccae* for use in the therapy of an autoimmune disease.

15. A pharmaceutical composition according to claim 14, which is sterile and pyrogen free.

16. A pharmaceutical composition according to claim 14 or 15, which is isotonic.

17. A pharmaceutical composition according to any one of claims 14 to 16, in unit dosage form.

18. A method substantially as hereinbefore described in any one of the examples.

Figure 1

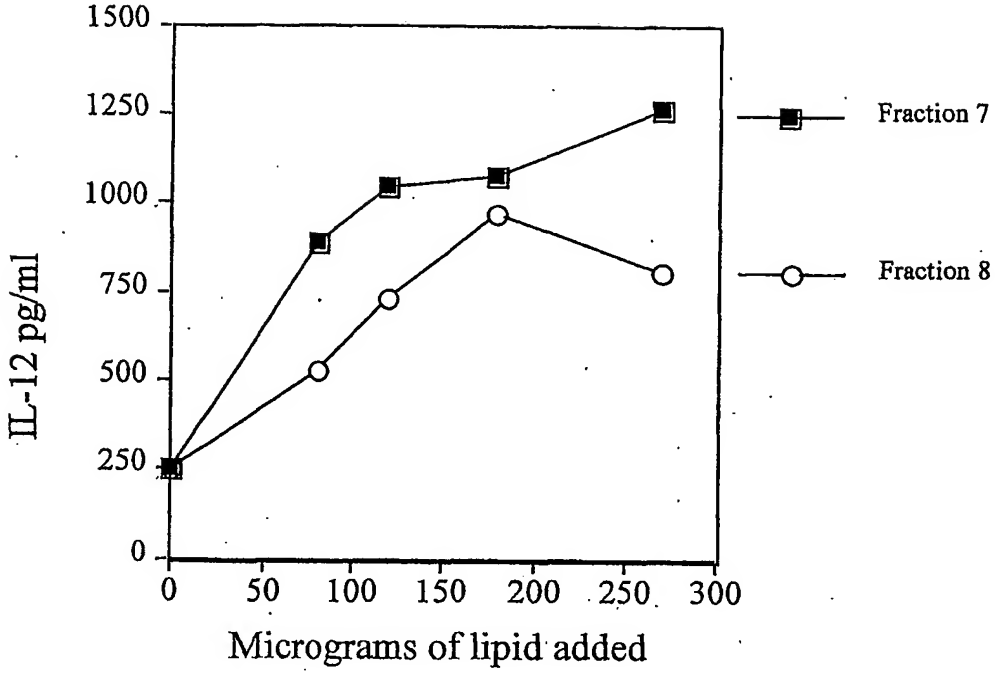
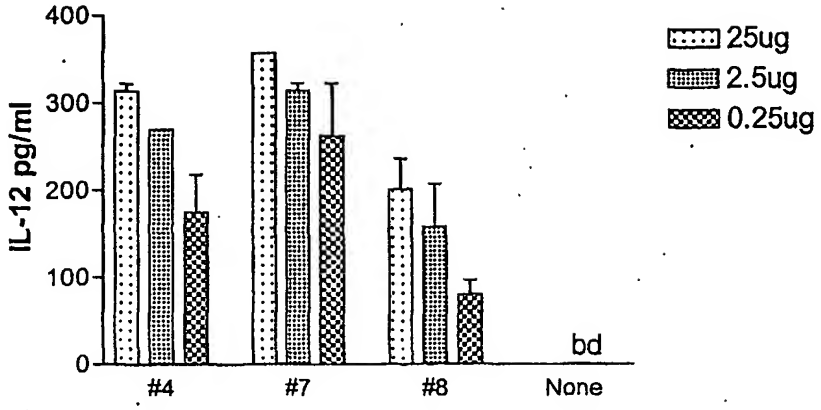

1500
1250
1000
750
500
250
0
IL-12 pg/ml
0
50
100
150
200
250
300
Micrograms of lipid added
Fraction 7
Fraction 8

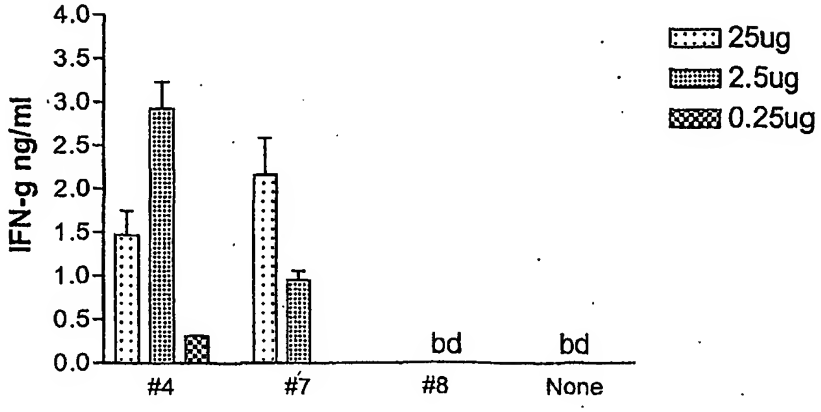
Figure 2

A



Lipid fraction added to normal mouse spleen cells

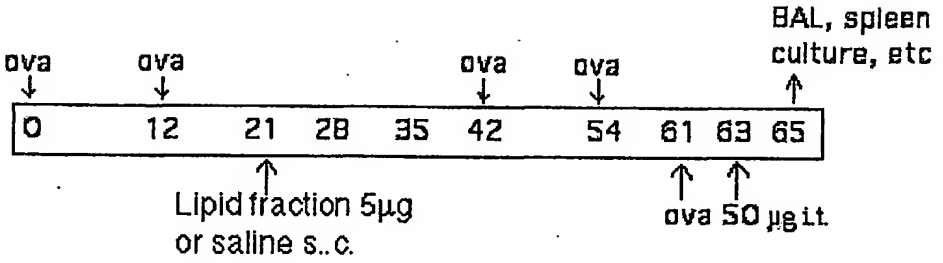
B



Lipid fraction added to normal mouse spleen cells

Figure 3 3/3

A



B